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Exclusion of Linkage Between RET and Neuronal Intestinal Dysplasia Type B

Virginia Barone, Daniel Weber, Yin Luo, Valeria Brancolini, Marcella Devoto, and Giovanni Romeo

Laboratorio di Genetica Molecolare, Istituto G, Gaslini, Genova, Italy (V.Ba., Y.L., G.R.); Department of Pediatric Surgery, Children's Hospital, Luzern, Switzerland (D.W.); and Department of Psychiatry, Columbia University, New York, New York (V.Br., M.D.)

Neuronal Intestinal Dysplasia type B (NID B) is a complex alteration of the enteric nervous system belonging to the group of intestinal dysganglionoses which may involve rectum, colon, and small intestine. Second only to Hirschsprung disease (HSCR), NID B is one of the most frequent causes of chronic constipation and pseudo-obstructive intestinal dysmotility. Since NID B is often associated with HSCR and point mutations in the RET proto-oncogene have been identified in HSCR patients, we analyzed two NID B pedigrees to investigate if RET mutations might cause also the NID B phenotype. Linkage analysis demonstrated that the NID B locus is not linked to RET in the pedigrees analyzed. Further genetic analyses will possibly improve the understanding of the cause and facilitate diagnostic procedures in NID B. © 1996 Wiley-Liss, Inc.

KEY WORDS: neuronal intestinal dysplasia, Hirschsprung disease, RET proto-oncogene

INTRODUCTION

In 1971 Meier-Ruge et al. described for the first time a disorder with impaired intestinal innervation and subsequently Fadda et al. [1983] differentiated two clinical and histochemical forms of Neuronal Intestinal Dysplasia (NID A and B). NID A is a very rare condition characterized by a congenital hypoplasia or aplasia of the sympathetic innervation of the intestine. Patients with NID A are infants with diarrhea, bloody stools, and intestinal spasticity. Their colons show mucosal inflammation and focal destruction of the muscularis mucosae. In NID B the parasympathetic submucous plexus is primarily affected. Characteristic histological and histochemical findings, on which the diagnosis is based, are 1) hyperplasia of submucosal plexuses with giant submucosal ganglia, 2) increased acetylcholinesterase activity in nerve fibers around submucosal blood vessels, 3) increased acetylcholinesterase activity in nerve fibers of the lamina propria mucosae, and 4) heterotopic ganglion cells in the lamina propria mucosae and in the muscularis mucosae [Borchard et al., 1991]. While most patients with NID B are sporadic, the observation of the few familial clusters suggested autosomal dominant inheritance [Schärli, 1992]. Different frequencies of NID B are reported, ranging from 1:4,000 to 1:60,000 live births [Martucciello et al., 1994]. This high variability may be due to difficulties in the diagnosis.

The clinical course of NID B is similar to that of intestinal aganglionosis: severe constipation, accompanied by intermittent abdominal distension developing within the first years of life with infrequent and painful defecation. Normally the clinical symptoms are independent of whether NID is localized or disseminated. Disseminated forms are rare and sometimes have a fatal course. Extreme cases may present with neonatal ileus. According to a published series, NID B is present in adult patients affected with chronic constipation and diverticulosis of the sigmoid colon [Stoss et al., 1994]. After diagnosis of NID B, children undergo conservative treatment for about 6 months. Persistent fecal retention, usually with absence of internal sphincter relaxation reflex, can often be successfully treated by posterior sphincteromyotomy. In case of massive rectal dilatation and absent propulsion, major surgery may be indicated.

Hirschsprung disease (HSCR) and NID B represent different forms of intestinal dysganglionosis: while in HSCR the intramural ganglion cells are absent in the aganglionic tract of the hindgut, NID B is characterized by hyperplasia of the submucous plexus of the colon with giant intramural ganglia. Both of these intestinal defects seem to be due to migration and maturation defects of primitive enteric neuroblasts deriving from neural crest cells [Martucciello et al., 1995; Holschneider et al.,

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Address reprint requests to Prof. Giovanni Romeo, Lab. Genetica Molecolare, Ist. G. Gaslini, L.go G. Gaslini 5, 16148 Genova, Italy.

1994]. Recently several point mutations in the RET proto-oncogene, which encodes for a receptor tyrosine kinase and is located at 10q11.2, have been identified in patients with HSCR [Romeo et al., 1994; Edery et al., 1994]. The expression of RET in cells derived from the neural crest and the frequent association of HSCR with NID B indicated the possibility that mutations in RET might be responsible also for the NID B phenotype. Therefore we performed a linkage study in two large NID B pedigrees with microsatellites tightly linked to the *RET* locus.

PATIENTS AND METHODS

At least one individual from each family (filled symbols in Fig. 1) was diagnosed using acetylcholinesterase (AChE) and lactate dehydrogenase (LDH) activities on suction and full-thickness biopsies from different segments of colon. All the patients who had an histological evaluation showed a localized recto-colonic NID B phenotype without aganglionosis. Other patients were diagnosed on the basis of clinical evidence only (hatched symbols). In Table I the clinical information on the members of these pedigrees are reported. In pedigree 1, individual III-5 (5 years old) had only little clinical evidence of NID B (hatched box). Individual III-4 (8 years old) of pedigree 2 also had hydrocephalus (star). The genotyping was determined using two microsatellites: sTCL-2 [Lairmore et al , 1993], which is located about 60 Kb from the 3 end of RET gene [Pasini et al., 1995a], and D10S141 [Gardner et al., 1993], which is located about 150 Kb centromeric with respect to sTCL-2. Genomic DNA was purified either from blood samples or from established lymphoblastoid cell lines with conventional methods. For each pair of primers, the primer having the higher melting temperature was labelled with γ^{32}P-dATP by T4 polynucleotide kinase. Standard PCR reactions were carried out with 0.4 μM of unlabeled primer and 0.1 μM of end-labeled primer in a total volume of 15 μl with 1–1.5 units of Taq polymerase.

Samples containing 100–200 ng of template DNA were initially denaturated at 94°C for 5 min; 30 cycles of amplification were performed by denaturing at 94°C for 40 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 30 sec, followed by a final step of 72°C for 7 min. Aliquots of 2–3 μl of PCR product were loaded on 6% polyacrylamide sequencing gels containing 7 M urea. Linkage analysis was performed using the MLINK program of the LINKAGE package version 5.2, assuming an autosomal dominant mode of inheritance. Disease allele frequency was calculated assuming a disease frequency of 1/4,000, and equal allele frequencies were assumed at the marker loci. Reduced penetrance was estimated from the same pedigrees by means of the ILINK program to be 0.70 for the heterozygous and homozygous carriers of the disease alleles. However, in order to avoid possible errors, all affected individuals were defined as affected, all spouses as unaffected, and all unaffected at risk of carrying the disease allele as unknown.

RESULTS

Figure 1 shows the two NID B pedigrees and the haplotype reconstruction for the two microsatellites analysed. In pedigree 1 and in pedigree 2 the two affected brothers (III-2 and III-3) and the two affected sisters (III-1 and III-3), respectively, have inherited different haplotypes from the carrier mother. In fact, as shown in Table II, two-point linkage analysis demonstrated that RET can be excluded as the candidate locus for NID B in each of our two families by the usual criterion of a negative lod-score of less than −2. In particular, we could exclude an interval of approximately 2 cM around the RET locus in each of the two pedigrees. If results from the two pedigrees were cumulated, under the hy-

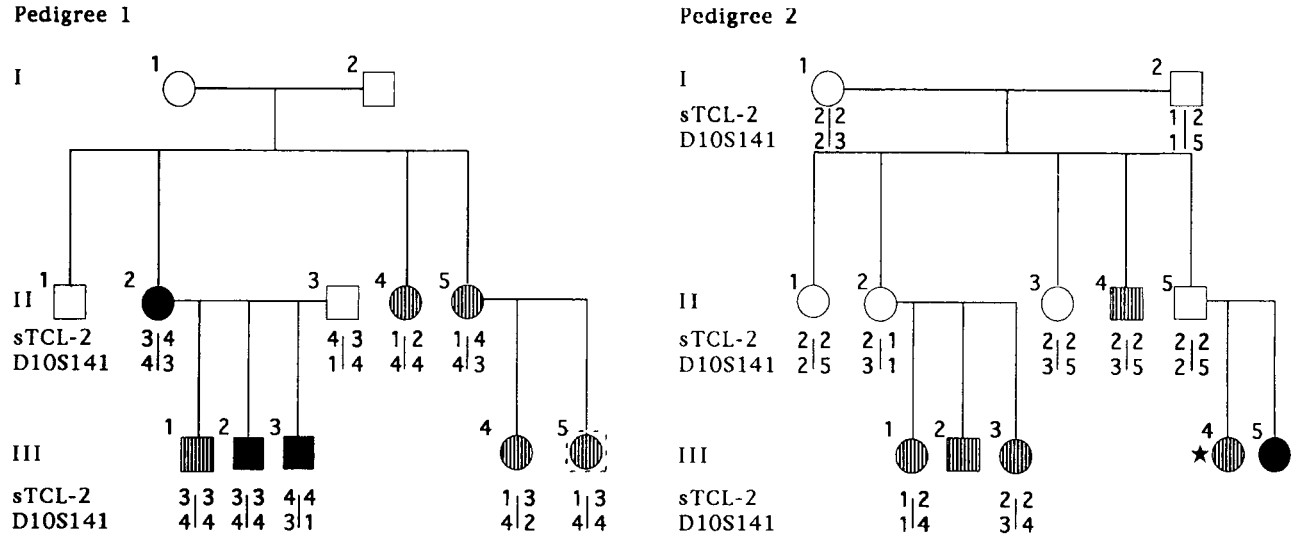

Fig. 1. NID B pedigrees and haplotype reconstruction of microsatellites sTCL-2 and D10S141. Filled symbols represent individual diagnosed both clinically and histologically. Other patients were diagnosed on the basis of clinical evidence (hatched symbols). Individual III/5 of pedigree 1 had only little clinical evidence of NID B (hatched box): individual III/4 of pedigree 2 showed also hydrocephalus (star).

TABLE I. Clinical and Histochemical Data on NID B Patients

Individuals	Pedigree 1	Pedigree 2
II-2	Age at diagnosis: 29 years. Histochemical findings: hyperplasia of submucosal plexus, heterotopic ganglia in muscularis mucosae, button-like ganglia	Healthy
II-3		Healthy
II-4	Massive, recurrent, postprandial emesis in infancy. Later periods of constipation followed by periods of diarrhea	At the age of 16 years abdominal pain. Investigations showed malfixation of the descending colon. At present massive constipation
II-5	Chronic constipation until her first pregnancy at 28 years. At present: daily 3–4 defecation of barely formed stool	Chronic constipation
III-1	Vomiting from the first year of life and fundoplicatio. Mild constipation	Defecation only when stimulated rectally. At the age of 3 years normalisation
III-2	Age at diagnosis: 2 years and 4 months. Histochemical findings: increased AChE in submucosal vessels, heterotopic ganglia in muscularis mucosae	Postprandial abdominal spasms from the first year of life. Normal defecation at the age of 4 years
III-3	Age at diagnosis: 1 year and 2 months. Histochemical findings: increased AChE in submucosal vessels, heterotopic ganglia in muscularis mucosae, button like ganglia	Healthy
III-4	Postprandial vomiting in infancy. From the age of 2 years and 6 months massive constipation	Stools always liquid
III-5	Period of mild constipation between 2 and 4 years	Age at diagnosis: 5 weeks. Histochemical findings: hyperplasia of submucosal plexus, heterotopic ganglia in muscularis mucosae, button like ganglia

pothesis that the same gene is responsible for the disease in both families, the exclusion interval became approximately 6 cM wide.

DISCUSSION

Severe constipation is a frequent disturbance in childhood. HSCR is a genetic disorder that explains many cases of fecal retention while among the remaining patients many show histological findings of NID B. HSCR and NID B represent two different forms of intestinal dysganglionosis due to maturation and/or migration defects of primitive enteric neuroblasts deriving from neural crest cells and are often found in association in the same patient and within the same family [Meier-Ruge et al., 1994]. The human proto-oncogene RET encodes for a receptor tyrosine kinase whose ligand is still unknown. Expression studies [Pachnis et al., 1993] during mouse embryogenesis and knock-out experiments [Schuchardt et al., 1994] demonstrated that RET is involved in neural crest development and in kidney organogenesis. Recently several mutations in the RET proto-oncogene have been identified in patients with HSCR [Romeo et al., 1994; Edery et al., 1994], causing a loss of function in two different expression systems [Pasini et al., 1995b]. In addition, since most NID B patients are sporadic, the observation of the two large pedigrees reported here led us to investigate whether the RET proto-oncogene, which is involved in HSCR, might be responsible also for the NID B phenotype. To this end we analysed the members of these families with two microsatellites tightly linked to the RET locus. Our results indicate that RET is excluded as the candidate gene for NID B at least in these 2 pedigrees. NID B could therefore be due to alteration(s) in other gene(s) involved in the enteric parasympathetic innervation. The same gene(s) might be involved also in HSCR, which is a genetically heterogeneous disease [Puffenberger et al., 1995; Attié et al., 1995; Yin et al., 1994]. While the clinical and histochemical findings of NID B are well known, little data

TABLE II. Two-Point Linkage Analysis Results Obtained in the NID B Pedigrees

	Recombination fraction						
Locus	0.0	0.01	0.05	0.1	0.2	0.3	0.4
sTCL-2							
Ped. 1	−5.38	−2.48	−1.18	−0.67	−0.25	−0.09	−0.02
Ped. 2	−0.48	−0.46	−0.40	−0.32	−0.18	−0.08	−0.02
Total	−5.86	−2.95	−1.58	−0.99	−0.43	−0.17	−0.04
D10S141							
Ped. 1	−2.20	−0.76	−0.17	0.01	0.07	0.04	0.01
Ped. 2	−5.68	−2.76	−1.43	−0.88	−0.39	−0.15	−0.04
Total	−7.88	−3.52	−1.60	−0.87	−0.32	−0.12	−0.03

are available on the cause of the disease. Furthermore, there are very few large pedigrees with recurrence of NID B, like the ones reported in the present work, which allow linkage mapping in this disease. Further genetic analyses might not only improve the understanding of the cause but also facilitate the development of diagnostic procedures and improve therapeutical approaches which should be based on a correct, although often difficult, diagnosis of NID B.

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REFERENCES

Attié T, Till M, Pelet A, Edery P, Bonnet JP, Munnich A, Lyonnet S (1995): Exclusion of RET and PAX 3 loci in Waardenburg-Hirschsprung disease. J Med Genet 32:312–313.

Borchard F, Meier-Ruge W, Wiebecke B, Briner J (1991): Innervationsstörungen des Dickdarms-Klassifikation und Diagnostik. Pathologe 12:171–174.

Edery P, Lyonnet S, Mulligan LM, Pelet A, Dow E, Abel L, Holder S, Nihoul-Fékété C, Ponder BAJ, Munnich A (1994): Mutations of the RET proto-oncogene in Hirschsprung's disease. Nature 367:378–379.

Fadda B, Maeir WA, Meier Ruge W, Schärli A, Daum R (1983): Neuronale intestinale Dysplasie. Eine kritische 10-Jahres-Analyse klinischer und bioptischer Diagnostik. Z Kinderchir 38:305–311.

Gardner E, Papi L, Easton DF, Cummings T, Jackson CE, Kaplan M, Love DR, Mole SE, Mulligan LM, Norum RA, Ponder MA, Reichlin S, Stall G, Telenius H, Telenius-Berg M, Tunnacliffe A, Ponder BAJ (1993): Genetic linkage studies map the multiple endocrine neoplasia type 2 loci to a small interval on chromosome 10q11.2. Hum Mol Genet 2:241–246.

Holschneider AM, Meire-Ruge W, Ure BM (1994): Hirschsprung's disease and allied disorders: A review. Eur J Pediatr Surg 4:260–266.

Lairmore TC, Dou S, Howe JR, Chi D, Carlson K, Veile R, Mishra SK, Wells SA Jr, Donis-Keller H (1993): A 1.5-Mb yeast artificial chromosome contig from human chromosome 10q11.2 connecting three genetic loci (RET, D10S94, and DS102) closely linked to the MEN 2A locus. Proc Natl Acad Sci USA 90:492–496.

Martucciello G, Caffarena PE, Lerone M, Mattioli G, Barabino A, Bisio G, Jasonni V (1994): Neuronal intestinal dysplasia: Clinical experience in Italian patients. Eur J Pediatr Surg 4:287–292.

Martucciello G, Favre A, Takahashi M, Iasonni V (1995): Immunohistochemical localization of RET protein in Hirschsprung disease. J Pediatr Surg 30:1–3.

Meier-Ruge W (1971): Über ein Erkrankugsbild des Colon mit Hirschsprung-Symptomatik. Verh Dtsch Pathol 55:506–510.

Meier Ruge R, Gambazzi F, Kaufeler RE, Schmid P, Schmidt ChP (1994): The neuropathological diagnosis of neuronal intestinal dysplasia (NID B). Eur J Pediatr Surg 4:267–273.

Pachnis V, Mankoo B, Costantini F (1993): Expression of the c-ret proto-oncogene during mouse embryogenesis. Development 119: 1005–1017.

Pasini B, Hofstra RMW, Yin L, Bocciardi R, Santamaria G, Grootscholten PM, Ceccherini I, Patrone G, Priolo M, Buys CHCM, Romeo G (1995a): The physical map of the human RET proto-oncogene. Oncogene, in press.

Pasini B, Borello MG, Greco A, Bongarzone I, Luo Y, Mondellini P, Alberti L, Miranda C, Arighi E, Bocciardi R, Seri M, Barone V, Radice MT, Romeo G, Pierotti MA (1995b): Loss of function effect of RET mutations causing Hirschsprung disease. Nature Genetics 10:35–40.

Puffenberger EG, Hosoda K, Washington SS, Nakao K, deWit D, Yanagisawa M, Chakravarti A (1995): A missense mutation of the Endothelin-B receptor gene in multigenic Hirschsprung's disease. Cell 79:1257–1266.

Romeo G, Ronchetto P, Yin L, Barone V, Seri M, Ceccherini I, Pasini B, Bocciardi R, Lerone M, Kääriäinen H, Martucciello G (1994): Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. Nature 367:377–378.

Schärli AF (1992): Neuronal intestinal dysplasia. Pediatr Surg Int 7: 2–7.

Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V (1994): Defects in kidney and enteric nervous system of mice lacking the tyrosine kinase receptor ret. Nature 367:380–383.

Stoff F, Meier-Ruge W (1994): Experience with Neuronal Intestinal Dysplasia (NID) in Adults. Eur J Pediatr Surg 4:298–302.

Yin L, Barone V, Seri M, Bolino A, Bocciardi R, Ceccherini I, Pasini B, Tocco T, Lerone M, Cywes S, Moore S, Vanderwinden JM, Abramowicz MJ, Kristoffersson U, Larsson LT, Hamel BCJ, Silengo M, Martucciello G, Romeo G (1994): Heterogeneity and low detection rate of RET mutations in Hirschsprung disease. Eur J Hum Genet 2:272–280.